Adenylyl cyclase G (ACG) is activated by high osmolality and mediates inhibition of spor germination by this stress factor. The catalytic domains of all eukaryote cyclases are active as dimers and dimerization often mediates activation. To investigate the role of dimerization in ACG activation, we coexpressed ACG with an ACG construct that lacked the catalytic domain (ACG\textsuperscript{cat}) and was driven by a UV-inducible promoter. After UV induction of ACG\textsuperscript{cat}, cAMP production by ACG was strongly inhibited, but osmostimulation was not reduced. Size fractionation of native ACG did not alter the dimer/monomer ratio. This indicates that ACG activity requires dimerization via a region outside the catalytic domain but that dimer formation does not mediate activation by high osmolality. To establish whether ACG required auxiliary sensors for osmostimulation, we expressed ACG cDNA in a yeast adenylyl cyclase null mutant. In yeast, CAMP production by ACG was similarly activated by high osmolality as in \textit{Dictyostelium}. This strongly suggests that the ACG osmosensor is intramolecular, which would define ACG as the first characterized primary osmosensor in eukaryotes.

INTRODUCTION

Fluctuations in external osmolality are one of the most commonly encountered stress signals of living cells. In prokaryotes, osmotic up-shifts activate transporters, such as ProP, BetP, and OpuA, which increase cytosolic solute levels. In addition, dual-component histidine kinases, such as KdpK and EnvZ are activated, which trigger transcription of transporter genes. Osmotic down-shifts trigger opening of mechanosensitive channels, such as MscL, and release of solutes. All these proteins harbor intramolecular osmosensors that either detect changes in membrane tension or in cytosolic ion concentrations. Such changes are the consequences of the passive water fluxes that follow osmotic shifts (Blount \textit{et al}, 1996; Jung \textit{et al}, 2000, 2001; van der Heide and Poolman, 2000; Racher \textit{et al}, 2001; Rubenhagen \textit{et al}, 2001).

In eukaryotes, the yeast dual component histidine kinase SLN1 is the best characterized osmoregulated protein. Phosphorelay initiated by the SLN1 histidine kinase inhibits the mitogen-activated protein (MAP) kinase HOG1, which triggers synthesis of the solute glycerol. Osmotic up-shift inhibits SLN1 kinase activity, which then allows HOG1 activation and glycerol synthesis to occur (Maeda \textit{et al}, 1994). In \textit{Arabidopsis}, the histidine kinase ATHK1 functions in a similar manner as SLN1 to activate a MAP kinase pathway after osmotic up-shift (Urao \textit{et al}, 1999). MAP kinase pathways also mediate osmotic stress responses in animals, but no primary osmosensors have yet been identified (Kultz and Burg, 1998). It is also not clear whether SLN1 or ATHK1 harbor an intramolecular osmosensor.

In the social amoeba \textit{Dictyostelium discoideum}, osmotic up-shift increases CAMP levels by two separate pathways. In the amoeba stage, osmotic up-shift reverts the histidine kinase DokA into a histidine phosphatase, which, similar to SLN1 and ATHK1, then acts as a phosphoryl group sink. This results in inactivation of the cAMP phosphodiesterase RegA and ultimately in fortification of the cell cortex (Schuster \textit{et al}, 1996; Zischka \textit{et al}, 1999; Ott \textit{et al}, 2000). DokA is a soluble protein that is indirectly regulated by osmotic up-shift through phosphorylation of a serine in the histidine kinase domain (Oehme and Schuster, 2001). In the spore stage, osmotic up-shift activates adenylyl cyclase G (ACG), an enzyme that is structurally homologous to the \textit{Trypanosoma} receptor adenyl cyclase (Ross \textit{et al}, 1991; Pitt \textit{et al}, 1992; Van Es \textit{et al}, 1996). High osmolality prevents premature germination of spores, while still in the fruiting body (Cotter and Raper, 1966; Virdy \textit{et al}, 1999), and this process is mediated by ACG acting on cAMP-dependent protein kinase (Van Es \textit{et al}, 1996).

The catalytic domains of eukaryote adenylyl- and guanylyl cyclases can only be active as dimers (Tesmer \textit{et al}, 1997; Zhang \textit{et al}, 1997). Although the catalytic domains can sometimes dimerize in isolation (Zhang \textit{et al}, 1997; Taylor \textit{et al}, 1999), the signaling processes that activate these enzymes usually act on regions elsewhere in the protein to either induce dimerization or to correctly juxtapose or stabilize the monomer partners (Hurley, 1998; Yu \textit{et al}, 1999).

We investigated the role of ACG dimerization in osmoregulation of enzyme activity, and we addressed the question whether the osmosensor is intrinsic to the ACG protein.

MATERIALS AND METHODS

ACG Expression in Yeast

\textit{Preparation of ACG cDNA.} An ACG cDNA was prepared from vector pBAGG (gift from Peter N. Devreotes, Johns Hopkins University School of Medicine, Baltimore, MD), which contains a 3.44-kb genomic fragment with the complete ACG coding sequence, 51 nucleotides (nt) 5′-untranslated region (UTR) and 616 nt 3′ UTR cloned into EcoRV-ClaI digested pBlueScript SK. The

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Table 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>cACG1</td>
<td>AGAGAGATCTCAAAAAC</td>
</tr>
<tr>
<td>cACG2</td>
<td>GACCTCATTTGAGCCG</td>
</tr>
<tr>
<td>HindIII-pBS5′</td>
<td>CACAAACGTCTCGGGCTGGAATTCG</td>
</tr>
<tr>
<td>SacI-ACG3′</td>
<td>GAGGGAACCTACAAGGAATGGCTAGTGG</td>
</tr>
<tr>
<td>BamHI-PrA5′</td>
<td>GAACTCGACGAAATCCGCGTTCATTCGTGGCC</td>
</tr>
<tr>
<td>Xhol-PrA3′</td>
<td>AAAGGATCCCAAACATTGTGAAATACTATC</td>
</tr>
<tr>
<td>BamHI-N-ACG</td>
<td>AACCTCAGAGTCTAAAAAGAAGCACAAGC</td>
</tr>
<tr>
<td>Xhol-ACG</td>
<td>AACCTCGAGCTTTTTTGTACATTCG</td>
</tr>
</tbody>
</table>

Xhol site in the multiple cloning site of pBAGC was eliminated by excision of the surrounding SacI/BamHI fragment, treatment with T4 DNA polymerase to blunt the 3′ and 5′ overhangs, and religation of the vector. The BgII/Xhol fragment from the ACG gDNA, which contains the two introns (Pitt et al., 1992), was then replaced by BgII/Xhol digested cDNA, which was generated by reverse transcription-polymerase chain reaction of *D. discoideum* spore RNA with primers cACG1 and cACG2 (Table 1). This yielded vector pBAGC.

**GALI-ACG Construct and Yeast Transformation.** An ACG gDNA fragment consisting of 511 of 5′ UTR and 2049 base pairs of ACG open reading frame was amplified from pBAGC by using primers HindIII-pBS5′ and SacI-ACG3′ (Table 1). This amplified product lacks the last 528 base pairs of the C-terminal region, which contains highly AT-rich repetitive sequence and may be recombinantly translated, this DNA fragment (which contains the two introns) will yield the 401 amino acids of the ACG gene but will lack almost the entire catalytic domain and the low-complexity C terminus. The first 401 amino acids of the ACG gene will be spliced out of the surrounding catalytic domain sequence and spliced into the GAL1-ACG construct (Table 1); this construct integrity was verified by DNA sequencing. The construct (GALI-ACG-ZZ) was transformed by means of the lithium acetate method (Gietz et al., 1992) into the null mutant TC41F2 (Pitt et al., 1992). Exponentially growing yeast cells were harvested from YPD medium (Burke et al., 1992), washed, and resuspended in 5 mM MES buffer, pH 6.0, at 30 mg of wet weight per milliliter. Cells were preincubated and subsequently stimulated with NaCl or sorbitol at 30°C as indicated in the figure legends. At different time intervals, 2.5-ml aliquots of cell suspension were quenched by rapid submerison in 10 ml of 60% methanol, precooled to -40°C, and pellets were centrifuged for 5 min at 1500 × g and 1°C and resuspended in 0.5 ml of 1 M perchloric acid. The suspension was transferred to a Microfuge tube containing 0.5 ml of glass beads (size 425–600 μm), vortexed for 10 cycles of 30 s at 4°C, and centrifuged for 5 min at 16,000 × g. The 20-μl aliquots of supernatant were neutralized with 8 μl of 100% saturated KHC03, and cAMP levels were measured by isotope dilution assay (Gilman, 1972; Thevelein et al., 1987a).

**Assay for cAMP Accumulation in Yeast.** Exponentially growing yeast cells were harvested from YPD medium (Burke et al., 2000), washed, and resuspended in 5 mM MES buffer, pH 6.0, at 30 mg of wet weight per milliliter. Cells were preincubated and subsequently stimulated with NaCl or sorbitol at 30°C as indicated in the figure legends. At different time intervals, 2.5-ml aliquots of cell suspension were quenched by rapid submerison in 10 ml of 60% methanol, precooled to -40°C, and pellets were centrifuged for 5 min at 1500 × g and 1°C and resuspended in 0.5 ml of 1 M perchloric acid. The suspension was transferred to a Microfuge tube containing 0.5 ml of glass beads (size 425–600 μm), vortexed for 10 cycles of 30 s at 4°C, and centrifuged for 5 min at 16,000 × g. The 20-μl aliquots of supernatant were neutralized with 8 μl of 100% saturated KHC03, and cAMP levels were measured by isotope dilution assay (Gilman, 1972; Thevelein et al., 1987a).

**Activation of the RNR Promoter and Assay for ACG Activity in Dictyostelium Cells.** Cells grown to 5 × 106 cells/ml in HL5 were irradiated with UV at 30 J/m2 by using a Bio-Link UV cross linker (VWR, Poole, United Kingdom). Cells were then diluted 10-fold in HL5 and left for 1 h at 22°C to allow transcription from the RNR promoter (Gaudet et al., 2003). The uninduced controls were treated in the same manner, except that UV irradiation was omitted. For assay of ACG activity, cells were washed free from HL5, resuspended to 5 × 106 cells/ml in KK2, and shaken for 10 min at 165 rpm. Aliquots of 25 μl of cell suspension were incubated with different variables in a total volume of 30 μl. Reactions were started by addition of the phosphodiesterase (PDE) inhibitor dithiobetitol to a final concentration of 5 mM and terminated by the addition of 30 μl of 35% perchloric acid (vol/vol). Lysates were neutralized with 50% saturated KHC03, and cAMP levels were measured by isotope dilution assay.

**Protein Fractionation.** To extract total *Dictyostelium* proteins under denaturing conditions, cells were resuspended to 2 × 106 cells/ml in KK2, mixed with an equal volume of 2× SDS-PAGE sample buffer, boiled for 5 min, and size fractionated on 10% SDS-PAA gels. The proteins were transferred to nitrocellulose membrane, and Western blots were incubated overnight at 4°C with a 1:2000 diluted homoser- ish peroxidase-conjugated goat anti-rabbit IgG (Promega, Madison, WI) as secondary antibody. To extract yeast proteins, 5 × 106 cells were resuspended in 1.2 ml of 10 mM Tris-HCl, pH 7.4, containing 0.3 M sorbitol, 0.1 M NaCl, 5 μM MgCl2, and 1× Complete protease inhibitor cocktail (Roche Diagnostics, Lewes, United King- dom) and vortexed at 4°C for 15 cycles of 30 s with 0.7 g of glass beads. Lysates were centrifuged at 800 × g for 4 min. Supernatants were mixed with an equal volume of 2× sample buffer and further treated as *Dictyostelium* total protein extracts.

**Assay for Dimer Formation.** To test for effects of osmotic up-shift on ACG dimer formation, cells were resuspended in KK2 and incubated for 5 min with or without 100 μM NaCl before being lysed by passage through nucleopore filters (pore size 3 μm). Lysates were prepared in the presence of 1× Complete protease inhibitor cocktail (Roche Diagnostics) and centrifuged for 30 min at 20,000 × g and 4°C. Pellets were washed in 5 mM glycine, pH 7.4, and centrifuged once more at 21,000 × g and 4°C.
Heterologous Expression of ACG

To investigate whether high osmolality acts directly on ACG or requires a separate sensor protein, we expressed ACG in the fungus *S. cerevisiae*, which is only distantly related to the mycetozoan *Dictyostelium*, which is only distantly related to the *S. cerevisiae* (Baldauf et al., 2000). To avoid interference from the yeast adenylyl cyclase, we used strain TC41F2-1, which carries a null mutation in the *CYR1* gene, that encodes the single yeast adenylyl cyclase (Heideman et al., 1990). *CYR1* is essential for growth and the cyr1 null mutant can only grow in the presence of 1 mM cAMP. An ACG cDNA was prepared in which 175 aa of C-terminal low-complexity sequence was replaced by a 140-aa protein region with the ZZ tag.

Expression of the ACG fusion construct was confirmed by immunoblotting of size-fractionated cell lysates with an αACG antibody. The antibody was raised in rabbit against a 19-aa peptide in the C-terminal region of ACG (Figure 1) and was tested for specificity on *aca* null cells and *aca* null cells that constitutively express ACG (Pitt et al., 1992). The antibody identified a single band of ~100 kDa on Western blots of *aca*/*ACG* cells, which agrees with the predicted size of 98 kDa for full-length ACG. The band was absent from *acg* null mutants, which indicates that the antibody is specific for ACG (Figure 2B). The yeast *cyr1* cells transformed with the GAL1-ACG-ZZ construct (*cyr1*/ACG) showed a somewhat smaller band, which was absent from the untransformed *cyr1* cells (Figure 2B). The difference in size is due to replacement of the C-terminal low-complexity region with the ZZ tag.

We measured whether cAMP production by ACG in the *cyr1*/ACG cells was stimulated by high osmolality. *Dictyostelium* cells secrete most of the cAMP that they produce and cAMP production by ACG can be readily detected in the extracellular medium. Under those conditions solute concentrations of 200 milliosmolar induce a further two- to fourfold stimulation of cAMP accumulation (Van Es et al., 1996). In contrast, yeast cells secrete very little cAMP and require rigorous procedures to break the cell wall before cAMP can be measured (Thevelein et al., 1987a). *cyr1*/ACG cells and the yeast wild-type strain *AY297* were stimulated with either 0.5 M NaCl or 1 M sorbitol or with low-osmolality buffer, and cAMP levels were measured at the indicated time intervals. Figure 3A shows that both 0.5 M NaCl and 1 M sorbitol induced a rapid increase of cAMP levels, which peaked at 45 s after stimulation. The osmolality required for maximal ACG stimulation in yeast was at 1 M rather higher than in *Dictyostelium* (Figure 3B). This could be due to dissimilarities in the phospholipid composition of *Dictyostelium* and yeast membranes, as was demonstrated for the bacterial osmosensor BetP (Rubenhagen et al., 2000). In wild-type yeast, both NaCl and sorbitol induced a decrease in cAMP.
normal development (Hopper et al., 1992). Wild-type and transformed spores either received a 45°C heat shock for 30 min or were left at 22°C before being incubated for 11 h in the presence and absence of 250 mM sucrose. Every 2 h, the ratio of spores to emerged amoebae was counted in a sample size of 100 cells. Means and SD of four experiments are presented.

**The Role of Dimerization in Osmoregulation of Spore Dormancy**

Truncated forms of the receptor guanylyl cyclases that lack the catalytic domain act as dominant-negative inhibitors because they sequester the native proteins by dimerization (Chinkers and Wilson, 1992). We used the same strategy to establish whether dimerization outside the catalytic domain is essential for ACG function during spore germination. Wild-type cells were transformed with a fusion construct of the psA prespore promoter and sequence encoding the N-terminal 401 amino acids of ACG. This construct (psA-ACGΔcat) lacks the cyclase domain and the low-complexity C-terminal region (Figure 1). The psA promoter directs expression of the construct in prespore and spore cells during normal development (Hopper et al., 1993).

*Dictyostelium* spores are normally activated to germinate by exposure to food, but rapid synchronous activation can be achieved by a heat shock. After activation spores enter a lag phase of 1–2 h before swelling and emergence occur. Exposure to high osmolality during the lag phase will induce the spores to return to dormancy (Cotter and Raper, 1967; Virdy et al., 1999). Figure 4A shows that after heat shock, wild-type spores germinated within 10 h at low osmolality, but not at high osmolality or in the absence of heat shock. In contrast to wild-type spores, the psA-ACGΔcat spores did not require a heat shock to germinate and germination was not inhibited by high osmolality (Figure 4B). These data suggest that ACGΔcat inhibits ACG function during spore germination.

**Figure 4.** Effects of a truncated ACG protein on spore germination. Wild-type cells were transformed with a fusion construct of the psA prespore promoter and an ACG truncation that lacks the catalytic domain (psA-ACGΔcat). Wild-type and transformed spores either received a 45°C heat shock for 30 min or were left at 22°C before being incubated for 11 h in the presence and absence of 250 mM sucrose. Every 2 h, the ratio of spores to emerged amoebae was counted in a sample size of 100 cells. Means and SD of four experiments are presented.

**The Role of Dimerization in Osmoregulation of ACG Activity**

Spores are inaccessible for direct measurement of ACG inhibition by ACGΔcat. We therefore coexpressed ACG and ACGΔcat in growing cells. Heterologous protein expression presents the problem that the absolute amount of expressed protein is determined by the copy number of the transformation vector. Results that depend on stochastic interaction between two heterologously expressed proteins will for this reason suffer from unpredictable clone-to-clone variability. To circumvent this problem, we coexpressed ACG under control of the constitutive actin15 promoter (A15-ACG) (Pitt et al., 1992) with ACGΔcat under control of the UV-inducible ribonucleotide reductase promoter (RNR-ACGΔcat). The UV-inducible promoter allows modulation of the level of ACGΔcat in the same transformed cell line (Gaudet et al., 1999; Gaudet et al., 2001). The vectors that harbor the A15-ACG and RNR-ACGΔcat gene fusions carried cassettes for neomycin and blasticidin selection, respectively, to allow for simultaneous selection and were transformed into *aca* cells to prevent interference with cAMP production by ACA, which is expressed during early development (Pitt et al., 1992). The RNR promoter was activated by exposure of growing cells to UV light, followed by further incubation for 1 h in growth medium to allow for optimal induction of RNR-ACGΔcat gene expression. Pilot experiments showed that in the absence of RNR-ACGΔcat, UV irradiation had no effect on ACG activity.

Figure 5A shows that cAMP accumulation in the parent *aca* strain is very low. Cells cotransformed with A15-ACG and RNR-ACGΔcat accumulate ~40 pmol of cAMP/10⁷ cells over a 10-min period in the absence of UV induction of ACGΔcat expression. Hyperosmotic conditions, created by 100 mM NaCl induced a twofold stimulation of cAMP accumulation (Figure 5B). After induction of RNR-ACGΔcat expression by UV, cAMP production by A15ACG was inhibited, but 100 mM NaCl still induced a twofold stimulation (Figure 5C). These data show that expression of ACGΔcat reduces the absolute level of ACG activity but does not prevent stimulation of the enzyme by high osmolality. The inhibitory effect of ACGΔcat seems rather moderate in comparison with its complete block of osmoregulation of spore germination as shown in Figure 4. However, it should be realized that in the spore germination experiment only the endogenous ACG, with low expression levels (Pitt et al., 1992) needs to be inactivated, whereas in the double-experiments performed in triplicate are presented.

**Figure 5.** Effect of conditional ACGΔcat expression on osmoregulation of ACG. *aca* cells were double transformed with constructs A15-ACG and RNR-ACGΔcat. The A15 and RNR promoters in these constructs direct constitutive and UV-inducible transcription, respectively. Effects of 100 mM NaCl on cAMP accumulation were measured in untransformed *aca* cells (A), uninduced transformants (B), and UV-induced transformants (C). Means and SE of three experiments performed in triplicate are presented.

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transformed cells ACGΔcat competes with ACG expressed from the strong actin15 promoter.

**Immunological Detection of ACG Dimerization**

To demonstrate that ACG and ACGΔcat form homo- and heterodimers, we isolated the native protein complexes. Cells transformed with both the A15-ACG and RNR-ACGΔcat constructs were exposed to UV to induce ACGΔcat expression and both UV-treated and untreated cells were stimulated for 5 min with 100 mM NaCl and lysed. As controls, we also included the aca parent strain and aca cells that were transformed with A15-ACG alone. Membrane proteins were solubilized, and native proteins were size fractionated by gel electrophoresis and immunoblotted with αACG antibodies. The antibody can detect full-length ACG but not ACGΔcat because the epitope is in the truncated region (Figure 1).

Figure 6 shows that in aca cells transformed with A15-ACG and RNR-ACGΔcat, that were not exposed to UV, the ACG antibody detected bands around 100 and 200 kDa in a roughly 70:30% ratio. These bands most likely represent monomers and dimers of full-length ACG, which are 98 and 196 kDa, respectively. Membrane proteins that were denatured and therefore monomerized by boiling in SDS sample buffer (DN) did not show the dimer band at 200 kDa, which indicates that this band does not represent nonspecific reactivity of the ACG antibody with a 200-kDa protein. This is further confirmed by the absence of any bands in native preparations from untransformed aca cells. After UV-induction of RNR-ACGΔcat expression, the 200-kDa bands largely disappeared and a novel band of ~140 kDa occurred. This band most likely represents the heterodimer of full-length ACG (98 kDa) and ACGΔcat (46 kDa). The appearance of the 140-kDa band is not an artifact of the UV exposure, because cells that only contained the A15-ACG construct showed the 100- and 200-kDa bands in the presence and absence of UV exposure. In both UV-induced and uninduced cells the monomer/dimer ratio was not altered by stimulation with 100 mM NaCl, which suggests that high osmolality does not promote dimer formation.

**DISCUSSION**

**ACG Harbors an Intramolecular Osmosensor**

ACG is predominantly expressed in the spore stage (Pitt et al., 1992), where it mediates inhibition of spore germination by high osmolality (Van Es et al., 1996). However, when expressed from a constitutive promoter in the amoeba stage, the enzyme was also activated by high osmolality, which suggested that the osmosensor could be intramolecular (Van Es et al., 1996). Because we could not rule out that an accessory sensor protein was expressed throughout development, it was essential to measure ACG activity in a system that was unlikely to express such a sensor. The choice of organism was limited to protists, because metazoan cells require culture at high osmolality. Preferably, the organism of choice should not display any adenylly cyclase activity, which could interfere with the assay. We chose a mutant strain of the fungus *S. cerevisiae* that lacks the single adenylly cyclase gene CYR1. In contrast to ACG, yeast CYR1 is not a transmembrane protein (Mitts et al., 1990); it is stimulated by glucose and requires both the heterotrimeric G protein Gpa2 and the small G protein Ras2 for activity (Thevelein et al., 1987b; Colombo et al., 1998). Because ACG is not regulated by G proteins (Pitt et al., 1992), it is unlikely that the mechanisms that activate CYR1 would act on ACG. In both yeast and *Dictyostelium*, high osmolality modulates histidine kinase activity (Maeda et al., 1994; Schuster et al., 1996), but the *Dictyostelium* histidine kinase DokA plays no role in regulating ACG (Ott et al., 2000) and the yeast enzyme SLN1, which targets the HOG1 MAP kinase pathway, is therefore also unlikely to do so. In fact, responses to osmotic stress are very different in the two organisms. Yeast cells respond to high osmolality with synthesis of the compatible osmolyte glycerol, a process that is mediated by the HOG1 pathway (Maeda et al., 1994). *Dictyostelium* cells do not accumulate compatible osmolytes and respond instead by remodeling of the cell cortex, a process that involves phosphorylation of actin and myosin (Zischka et al., 1999). It is therefore unlikely that any component of the osmostimulated HOG1 pathway, including glycerol, would activate ACG.

*Dictyostelium* ACG complemented the growth defect of the yeast cyr1 null mutant (Figure 2) and similar to *Dictyostelium* cells, cAMP production in the cyr1/ACG cells was stimulated by high osmolality. However, higher solute concentrations were required and the stimulated cAMP accumulation in yeast was more transient than in *Dictyostelium*.

The transient kinetics of cAMP accumulation in yeast is probably due to the fact that the yeast cAMP phosphodiesterase PDE1 is strongly stimulated by cAMP through activation of cAMP-dependent protein kinase. This rapidly neutralizes glucose-induced cAMP accumulation in wild-type yeast after 45 s of stimulation (Ma et al., 1999), which agrees exactly with the kinetics of high osmolality-induced cAMP accumulation in cyr1/ACG cells (Figure 3A). This suggests that the transient kinetics of cAMP accumulation in yeast is a function of PDE1, rather than of either CYR1 or ACG. The pronounced effect of PDE1 on cAMP levels in yeast raises the possibility that high osmolality could have increased cAMP by inhibiting PDE1 (or PDE2), instead of by activating ACG. However, this cannot be the case because in wild-type...
Role of Dimerization

A wide range of signal transduction proteins ranging from growth factor receptors (Schlessinger, 2002), dual component histidine kinases (Bilwes et al., 1999), and STAT transcription factors (Imada and Leonard, 2000) to adenyl cyclases (Tesmer et al., 1997; Zhang et al., 1997) requires dimerization for activity, and in many cases, dimer formation mediates activation of these proteins. Eukaryote adenyl- and guanylyl cyclases can only bind substrate when two catalytic domains are combined in an antiparallel wreath-like structure (Liu et al., 1997). In the case of the G protein-regulated adenyl cyclases, which harbor two intramolecular catalytic domains, the monomers are already joined and activating factors such as Gs or forskolin promote the catalytically optimal juxtaposition of the two monomers (Hurley, 1998). However, for the single transmembrane cyclases, which like ACG harbor a single catalytic domain, dimerization can mediate activation. This is the case for the retinal guanylyl cyclase RetGC, which is dependent on guanylyl cyclase activator proteins (GCAPs) for dimer formation. Each RetGC monomer binds to a GCAP monomer. The GCAP monomers dimerize in the absence of cytosolic Ca"++, and this also brings the RetGC monomers together (Ol'shevskaya et al., 1999; Yu et al., 1999). In the receptor guanylyl cyclases, such as the atrial natriuretic peptide (ANP) receptor, activation is due to an increase in relative orientation of the dimers, rather than in dimer formation itself. In the basal state the ANP receptor forms an inactive dimer by interactions between the intracellular coiled-coil and kinase homology domains. The ligand ANP binds simultaneously to the extracellular regions of the two monomers, which triggers closure of their membrane proximal domains. This causes a change in alignment of the intracellular domains, which ultimately results in activation of guanylyl cyclase (Wilson and Chinkers, 1995; He et al., 2001; Labrecque et al., 2001).

ACG and the Leishmania and Trypanosoma adenyl cyclases show a superficial structural similarity to the ANP receptor, but no kinase homology domain or coiled-coil domains are present (Figure 7). There is no significant sequence similarity between the extracellular domains of ANP-R, ACG, and the parasite cyclases. The isolated catalytic domains of the T. brucei enzymes ESAG4.GRESAG4.4B and GRESAG4.3 show a weak tendency to form catalytically competent dimers (Bieger and Essen, 2001). The catalytic domains of both GRESAG4.4B and TczAC can form stable dimers, although the full-length TczAC protein shows much higher catalytic activity (D’Angelo et al., 2002). Enforced dimer formation by fusion of a leucine zipper to the GRESAG4.4B catalytic domain also greatly enhanced activity (Naula et al., 2001). In the case of ACG, the isolated catalytic domains showed no catalytic activity and did not dimerize (our unpublished data). This group of enzymes is therefore likely to either require additional proteins or additional regions within the protein to enforce the dimerized state.
We show here that the ACG\textsubscript{cat} construct without the catalytic domain can dimerize with the full-length protein (Figure 6) and acts as a dominant-negative inhibitor of enzyme activity (Figures 4 and 5). This indicates that in addition to the catalytic domain, ACG has a separate dimerizing region. Unlike ANP-R, neither ACG nor the parasite adenyl cyclases have a significant juxtamembrane intracellular region that could mediate dimer formation (Figure 7), and dimerization therefore most likely requires the extracellular domain of the protein.

Our data do not support a role for osmotic up-shift as direct trigger for dimerization. Overexpression of ACG\textsubscript{cat} in spores blocked the inhibition of spore dormancy by high osmolality, but the spores also no longer required a heat shock to activate the germination process (Figure 4). Spores contain 10 times higher cAMP concentrations than amebae, most of which is produced by ACG (Virdy et al., 1999). Shortly after heat shock cAMP levels decrease dramatically, but they transiently increase again during the postactivation lag phase. This increase is more pronounced when heat-activated spores are exposed to high osmolality. Heat shock induces a transient loss of ACG mRNA, which may cause the decrease of cAMP levels and activation of the germination process (Virdy et al., 1999). The fact that ACG\textsubscript{cat} both obviated the requirement for heat shock and prevented osmoregulation of germination suggests that it inhibits both basal and osmostimulated ACG activity. This was confirmed by measurement of ACG activity in the presence and absence of the ACG\textsubscript{cat}. Even though ACG\textsubscript{cat} strongly reduced ACG activity, it did not prevent activation of the residual activity by high osmolality (Figure 5). Most importantly, high osmolality did not increase the ACG dimer to monomer ratio (Figure 6). We therefore conclude that high osmolality does not activate ACG by causing it to dimerize and that osmoregulation of ACG involves a novel mode of regulation of this important class of enzymes.

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REFERENCES


